PHOTOCHEMICAL ACTION SPECTRUM OF THE CO-INHIBITED 5ß-CHOLESTANE-3α, 7α, 12α-TRIOL 26-HYDROXYLASE SYSTEM

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Received November 29,1976

SUMMARY

The inhibition of the mitochondrial hydroxylation of 5ß-cholestane-3α, 7α, 12α-triol at the 26 position by a $CO:O_2$ gas mixture was maximally reversed by monochromatic light at the wavelength of 450 nm. This establishes the involvement of a cytochrome P450 dependent monooxygenase in the 26-hydroxylation of 5ß-cholestane-3α, 7α, 12α-triol in rat liver mitochondria.

INTRODUCTION

It has been shown by Okuda et al. that the terminal methyl group of 5ß-cholestane-3α, 7α, 12α-triol, an intermediate in the conversion of cholesterol to cholic acid, is hydroxylated by rat liver mitochondria in the presence of NADPH or a rat liver boiled extract (1). Cronholm et al. (2), however, argued against the possible existence of the hydroxylase in mitochondria, attributing the mitochondrial activity observed by the previous authors to a microsomal contamination of the mitochondrial preparation. These workers found a relatively high 26-hydroxylase activity in microsomes together with hydroxylation activities at carbon atoms 23, 24 and 25. Contrarily, Taniguchi et al. (3) reconfirmed that the enzyme is actually present in rat liver mitochondrial inner membrane matrix by applying the

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Schnaitman's releasing technique of mitochondrial membrane with digitonin. Furthermore, they observed that the hydroxylation is inhibited in the presence of carbon monoxide, and postulated that a cytochrome P450 like entity may be responsible for the reaction. However, no detectable decrease of the inhibition due to carbon monoxide was observed on illumination with white light up to 21.5 klm/m^2. Conclusive evidence that the CO-binding entity in rat liver mitochondria is cytochrome P450, therefore, remained to be established.

In this communication we report the photochemical action spectrum of the 26-hydroxylase present in rat liver mitochondria which provides definite proof that the liver mitochondrial CO-binding entity is cytochrome P450.

MATERIALS AND METHODS

Uniformely labeled 5ß-cholestane-3α, 7α, 12α-triol was synthesized by the Kolbe reaction from cholic acid, which was randomly labeled according to the method of Wilzbach (Radiochemical Centre, Amersham, England). This was purified by aluminum oxide column chromatography using benzene:ethyl acetate as eluting solvent, and crystallized to a constant specific activity of 5.3 x 10^4 d.p.m./min./ng. Mitochondria were prepared from male Sprague-Dawley rats (120-150 g) according to the method described by Wilgram et al. (4). The experimental conditions for determination of the photochemical action spectrum were as follows: Incubations were carried out in a 1 cm glass cuvette fitted with a rubber septum pierced with two fine stainless steel needles. The reaction mixture, containing 50 nmoles [^{3}H] 5ß-cholestane-3α, 7α, 12α-triol dissolved in 10 µl of acetone, 2.6 µmoles $MgCl_2$, 1.5 µmoles KCN, 75 µmoles potassium phosphate buffer, pH 7.0 and water to make a final volume 1.5 ml, was equilibrated with a gas mixture (80 % CO and 20 % O_2) for 5 min. One hundred microliters of mitochondrial suspension (10-15 mg of protein/ml) were then introduced through one of the needles. The cuvette was placed in a cuvette holder maintained at 30°, and left to equilibrate for 5 min. with occasional shaking. The reaction was started by introducing 0.1 ml 20 mM DL-isocitrate The incubations were carried out for 10 min. in the dark or in a beam of monochromatic light of various wavelengths. The apparatus was essentially the same as that described in the

Table I

Reversal of CO-Inhibition by Lights of Various Wavelengths

Incubation conditions are given in the text. The cuvettes were gassed with a mixture of 80 % CO and 20 % O_2. Control incubations (without CO) were gassed with a mixture of 20 % O_2 and 80 % N_2. V_{CO} is the rate of 26-hydroxylation in the presence of CO. $n = V_{CO}/V_{O_2}$, where V_{O_2} is the rat of 26-hydroxylation in the absence of CO, and was 1.0 nmole/min. $K = (CO/O_2 \cdot (n/1-n)$. $\Delta K = K-K_d$, where K_d value is the value of K obtained in the dark. The light sensivity, $L = 1/i\ (\Delta K/K_d)$, where i is the intensity of the light beam striking the cuvette in mole quanta/cm^2min.

Wavelength (nm)	V_{CO} (nmoles/min)	n	K	ΔK	L/L_{450}
Dark	0.21	0.21	1.06		
410	0.49	0.49	3.84	2.78	0.151
422	0.62	0.62	6.53	5.46	0.258
430	0.52	0.52	4.33	3.27	0.110
450	0.90	0.90	36.00	34.94	1.000
470	0.46	0.46	3.41	2.34	0.068
492	0.33	0.33	1.97	0.91	0.035
500	0.30	0.30	1.71	0.65	0.025

previous report (5). The relative energy distribution of light at the various wavelenghts was measured by a compensated thermopile (Kipp and Zonen, CA 1). The reaction was terminated by adding 0.1 ml of 2 N HCl, and the mixture was extracted with 2 ml of ethyl acetate. The extract was washed with water and the solvent evaporated under reduced pressure. The residue was dissolved in ethyl acetate and subjected to thin layer chromatography using the solvent ethyl acetate: acetone, 70:30, v/v (6). The distribution of radioactivity on the plate was measured by a thin layer scanner (Berthold, Wildbad, G.F.R.). The conversion of 5ß-cholestane-3α, 7α, 12α-triol into the product, 5ß-cholestane-3α, 7α, 12α, 26-tetrol, was calculated from the peak area (3). O-dealkylation of 7-ethoxycoumarin was measured according to the method described by Ullrich et al. (7). Protein concentration was determined by the method of Lowry (8).

RESULTS AND DISCUSSION

In the presence of 80 % CO and 20 % O_2 ($CO:O_2$ ratio of 4.0) the hydroxylation of 5ß-cholestane-3α, 7α, 12α-triol by rat liver mitochondria containing DL-isocitrate

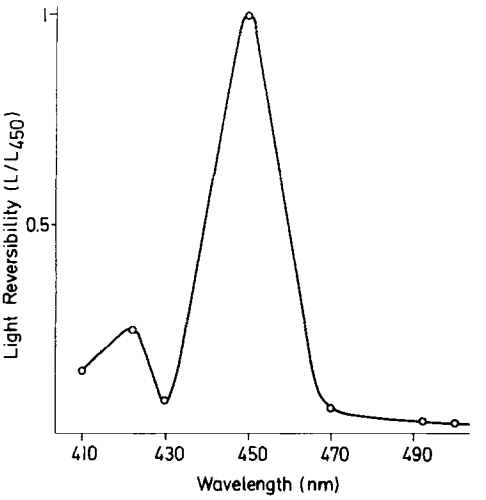

Fig. 1. Photochemical action spectrum of the reversibility of the CO-inhibited 5β-cholestane-3α, 7α, 12α-triol 26-hydroxylation. The relative light sensitivities, L/L_{450}, for each wavelength of light were obtained from Table I and plotted as a function of the wavelength.

was inhibited to about 80 % in the dark as shown in Table I. By irradiation with light of various wavelengths the inhibition was reversed to a different extent, light of 450 nm being most effective. The photochemical action spectrum thus obtained is shown in Fig. 1. Following the theory of Warburg (9) the spectrum was plotted in terms of relative light sensitivity against wavelength of acting light. As shown in the figure the photochemical action spectrum closely resembles the absolute spectrum of cytochrome P450-CO complex (10). This results gave positive evidence for the involvement of cytochrome P450 in the reaction pathway. A second peak was observed at about 420 nm, the significance of which is not at present known and requires further study. A similar observation was also reported by many others.

The extent of a possible contamination with microsomes of the mitochondrial preparation was estimated by measuring 7-ethoxycoumarin O-dealkylation activity. The specific activity of this enzyme activity in the mitochondrial preparation was 0.01 nmoles/mg of protein, which is about one fiftieth of that in the corresponding microsomal fraction (0.5 nmoles/mg of protein). The contamination of the mitochondrial preparation by microsomes was therefore at maximum 2 %. A possible contribution of these microsomes, to the 26-monooxygenation could however, be ruled out by using isocitrate as a donor. Isocitrate is not an electron donor to cytochrome P450 in microsomes. In fact Taniguchi et al. observed that microsomal hydroxylation activity of the steroid in the presence of NADPH was virtually lost when isocitrate was substituted for NADPH (3). The products obtained in the present experiment, therefore, seemed to be exclusively due to cytochrome P450 dependent monooxygenase present in mitochondria. Thus the photochemical action spectrum shown in Fig. 1 verifies conclusively that rat liver mitochondrial 26-hydroxylase involves a mitochondrial cytochrome P450 species.

ACKNOWLEDGEMENT

The authors wish to thank Dr. C.R. Wolf for his reading the manuscript and discussions. The support by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 38, is gratefully acknowledged.

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